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Molecular and Cellular Neuroscience 14, 419–427 (1999)
Article ID mcne.1999.0811, *available online at http://www.idealibrary.com on* IDEAL®

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Identification of a Novel Aspartic Protease (Asp 2) as β-Secretase

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The Alzheimer's disease β-amyloid peptide (Aβ) is produced by excision from the type 1 integral membrane glycoprotein amyloid precursor protein (APP) by the sequential actions of β- and then γ-secretases. Here we report that Asp 2, a novel transmembrane aspartic protease, has the key activities expected of β-secretase. Transient expression of Asp 2 in cells expressing APP causes an increase in the secretion of the N-terminal fragment of APP and an increase in the cell-associated C-terminal β- cretase APP fragment. Mutation of either of the putative catalytic aspartyl residues in Asp 2 abrogates the production of the fragments characteristic of cleavage at th β-secretase site. The enzyme is present in normal and Alzheimer's disease (AD) brain and is also found in cell lin s known to produce Aβ. Asp 2 localizes to the Golgi/ ndoplasmic reticulum in transfected cells and shows clear olocalization with APP in cells stably expressing the 751-amino-acid isoform of APP.

INTRODUCTION

Production and deposition of the 39- to 43-residue amyloid-β protein (Aβ) in the brain are early and invariant neuropathological features of Alzheimer's disease (Glenner and Wong, 1984). Aβ is produced by cleavage of the amyloid precursor protein (APP). APP is a substrate for α- or β-secretase activities which release soluble N-terminal fragments of the protein, sAPPα and sAPPβ (Selkoe, 1994). The resulting membrane-anchored C-terminal fragments (CTFα and CTFβ) are substrates for γ-secretase; cleavage of CTFα gives rise to the 3-kDa peptide p3 and cleavage of CTFβ gives rise to the 4-kDa Aβ peptide. The secretases involved in the processing of APP have not been isolated but inhibitor studies suggest that α-secretase is a metalloproteinase (Parvathy *et al.*, 1998). A number of candidate β-secretases have been proposed and subsequently eliminated; these include the proteasome (Ishiura *et al.*, 1989), the metalloproteinase thimet (McDermot *et al.*, 1992), several chymotrypsin-like serine proteases (Nelson *et al.*, 1993; Sahasrabudhe *et al.*, 1993; Savage *et al.*, 1994), the metalloproteinases MP78 (Thompson *et al.*, 1997) and MP100 (Huber *et al.*, 1999), and cathepsin D (Ladror *et al.*, 1994). Recently it has been reported that presenilin-1 is either a unique diaspartyl cofactor for γ-secretase or γ-secretase itself (Wolfe *et al.*, 1999). The β-secretase cleavage event has been shown to occur within specific compartments of the endomembrane system, including the rough endoplasmic reticulum and the trans-Golgi network (Hartmann *et al.*, 1997; Cook *et al.*, 1997; Wild-Bode *et al.*, 1997). Aβ is generated at a slow rate intracellularly prior to its secretion and an intraneuronal pool of Aβ has been reported that accumulates with time in cultured cells (Skovronsky *et al.*, 1998).

We have previously reported the identification and characterization of a novel transmembrane aspartyl proteinase which we have termed Asp 2 that shows high levels of expression in brain and pancreas (European Patent Applica-

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tion EP0855444, SmithKline Beecham). We show here that this enzyme has the key characteristics expected of β-secretase and conclude that this is the major cellular activity that catalyzes cleavage of APP at the β-site.

RESULTS

We have identified a novel membrane-bound aspartyl protease (Asp2) using a proprietary EST database, and subsequently cloned the full-length cDNA from a melanoma Marathon-Ready cDNA preparation (Clontech Laboratories, Inc.). The amino acid sequence of this protease is shown in Fig. 1a. The sequence features of Asp 2 indicate that it is a novel aspartic protease having a cleaved signal peptide, a prodomain segment, a catalytic domain that identifies it as an aspartic protease, a putative transmembrane domain, and a cytoplasmic carboxy-terminal domain (Figs. 1a and 1b). As stated in the Introduction, α- or β-secretase activities release soluble N-terminal fragments of APP (sAPPα and sAPPβ), leaving the membrane-anchored fragments (CTFα and CTFβ). Cleavage by γ-secretase of CTFα and CTFβ gives rise to the 3-kDa peptide p3 and to the 4-kDa Aβ peptide, respectively (Fig. 1c). The predicted topology of Asp 2 is shown in Fig. 1d and is based upon the presence of putative glycosylation sites in the amino-terminal domain and protease digestion studies of protein translated *in vitro* in the presence of dog pancreatic microsomes (data not shown).

Transient transfection of SH-SY5Y APP-695 cells with Asp 2 (Fig. 2a) results in a significant increase in the secretion of sAPPβ (Fig. 2b) consistent with Asp 2 being β-secretase. To demonstrate that this increase in sAPPβ is linked to the proteolytic activity of Asp 2, we mutated each of the proposed catalytic aspartic residues at positions 25 and 215 (determined by comparison with the position of the known catalytic aspartyl residues in pepsin) to asparagine. Both mutants and the wild-type Asp 2 are expressed to similar levels (Fig. 2a). However, expression of either of the Asp 2 mutants does not produce the increase in the secretion of sAPPβ (Fig. 2b) seen for wild-type Asp 2. In contrast to this clear effect on sAPPβ, Asp 2 has no effect on the secretion of soluble APPα or on full-length APP in the cell (data not shown).

Cathepsin D is an aspartic proteinase which has been shown to cleave a synthetic β-secretase cleavage peptide at the β-secretase site (Chevallier *et al.*, 1997). Therefore, as a further control, we transiently expressed cathepsin D in the SH-SY5Y APP-695 cells. Cathepsin D is expressed as a 52-kDa proenzyme and a 34-kDa form (Fig. 2c), which in conjunction with the 14-kDa fragment constitutes the enzymatically active proteinase. In contrast to the expression of Asp 2, cathepsin D does not cause an increase in the secretion of sAPPβ (Fig. 2d).

The production of distinct carboxy-terminal fragments is diagnostic for cleavage of APP at the β-secretase site (Selkoe, 1994). For example, expression of a β-secretase candidate would result in an increase in the level of CTFβ with no effect on the level of CTFα (Fig. 3a). To examine the production of these fragments we used an antibody (Ab54; Allsop *et al.*, 1997) that recognizes both CTFα and CTFβ and an antibody (WO2; Ida *et al.*, 1996) that specifically recognizes the carboxy-terminal fragment produced by β-secretase cleavage. Asp 2 was transiently transfected into COS-7 APP-751 cells and into COS-7 APP-751 cells stably expressing the Swedish mutation $KM^{651,652}NL$ (Haass *et al.*, 1997). The levels of Asp 2 expression are similar in all transfection experiments (Figs. 3b, 3e, and 3h) and the protein can be seen to migrate as a doublet consistent with glycosylation as described above. Cathepsin D again served as a control and appears as two bands (Fig. 3h). As shown in Figs. 3c, 3f, and 3i, 12- and 10-kDa bands are detected in both stable cell lines by Ab54 representing CTFβ and CTFα. However, the levels of CTFβ are significantly elevated in both cell lines when Asp 2 is transiently expressed (Figs. 3c, 3f, and 3i). In contrast, transient transfection of cathepsin D does not lead to an increase in the production of CTFβ (Fig. 3i). Using antibody WO2, which is specific for residues 5–9 of human Aβ, only the 12-kDa fragment is detected, thus confirming the identity of this fragment as the C-terminal fragment produced by the action of β-secretase (CTFβ). The 10-kDa fragment band is not immunoreactive with this antibody and this fact, combined with its molecular weight, identifies it as CTFα,

FIG. 1. An alignment of the amino acid sequences of Asp 2 with human pepsin, cleavage pathways of APP, and the proposed topology of Asp 2. (a) The predicted signal sequence is underlined at the N-terminus. The predicted beginning of the mature domains of Asp 2 is highlighted (() beside the known N-terminal residue of mature pepsin (()). The catalytic Asp residues (*) in the conserved catalytic DTG triads and the conserved Tyr residue (●) in the beta-hairpin loop (flap) of each enzyme are also highlighted. The predicted membrane-spanning domain of Asp 2 is underlined in each case. Potential N-linked glycosylation sites are underlined in italics. (b) Schematic structure of Asp 2. The numbers indicate amino acid positions. Yellow, signal sequence; red, prodomain; clear, catalytic domain; green, transmembrane domain; blue, cytosolic domain. Also shown are the conserved protease active-site domains. (c) APP can be cleaved by α, β, and γ-secretases. β-Secretase cleavage of APP gives rise to the secretion of the N-terminal domain sAPPβ and a 12-kDa C-terminal fragment (CTFβ). Subsequent cleavage of CTFβ by γ-secretase gives rise to Aβ. α-Secretase cleavage of APP gives rise to the secretion of the N-terminal domain sAPPα and a 10-kDa C-terminal fragment (CTFα). Subsequent cleavage of CTFα by γ-secretase gives rise to p3. (d) Asp 2 is a type 1 integral membrane protein with the catalytic domain being lumenal. Also shown is the location of the C-terminal *myc*-His tag.

a

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Asp2     M-AQALPWLLLNMGAGVL----PAHGTQHGIRLPLRSGLGG-----APL-GLRLP----R-ET   47
Pepsin   MKWLLLLGLVA-------------LSEGIMYKVPLI------KKKSFRRTLSERGLLKDFLK   49

Asp2     DE----EPEE--PGRRGSFVEMVDNLRGKSGQGYYVEMTVGSPPQTLNILVDTGSSNFAVG  102
Pepsin   KHNINPARKYFPQWKAPTLVDEQPLENYLDMEYFGTIGIGTPAQDFTVVFDTGSSNLWVP  109

Asp2     AAPH-----PFLHRYYQRQLSSTYRDLRKGVYVPYTQGKWEGELGTDLVSIPHGPNVTVRA  158
Pepsin   SVYCSSLACTNHNRFNPEDSSTYQSTSETVSITYGTGSMTGILGYDTVQVG--GISDTNQ  161

Asp2     NIAAITESD-KFFINGSNWEGILGLAYAEIARPDDSLEPFFDSLVKQTHV-PNLFSLQLC  216
Pepsin   -IFGLSETEPGSFLYYAPFDGILGLAYPSISS--SGATPVFDNIWNQGLVSQDLFSVYLS  218

Asp2     GAGFPLNQSEVLASVGGSMIIGGIDHSLYTGSLWYTPIRREWYYEVIIVRVEINGQDLKM  276
Pepsin   AD--DQ--S-------GSVVIFGGIDSSYYTGSLNWVPVTVEGYWQITVDSITMNGEAI--  266

Asp2     DCKEYNYDKSIVDSGTTNLRLPKKVFEAAVKSIKAASSTEKFPDGFWLGEQLVCWQAGTT  336
Pepsin   ACAEG--CQAIVDTGTSLLTGPTSPIANIQSDIGA----SENSDGDMVV---SCSAISSL  317

Asp2     PWNIFPVISLYLMGEVTNQSFRITIL-PQQYLRPVEDVATSQDDCYKFAISQ---SSTGT  392
Pepsin   P-------------------DIVFTINGVQYPVPPSAYILQSEGSCISGFQGMNLPTESGE  359

Asp2     --VMGAVIMEGFYVVFDRARKRIGFAVSACHVHDEFRTAAVEGPFVTLDMEDCGYNIPQT  450
Pepsin   LWILGDVFIRQYFTVFDRANNQVGLAPVA*                                 388

Asp2     DESTLMTIAYVMAAICALFMLPLCLMVCQ-WRCLRCLRQQHDDFADDISLLK*          501
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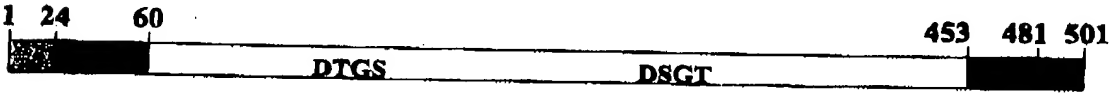
b



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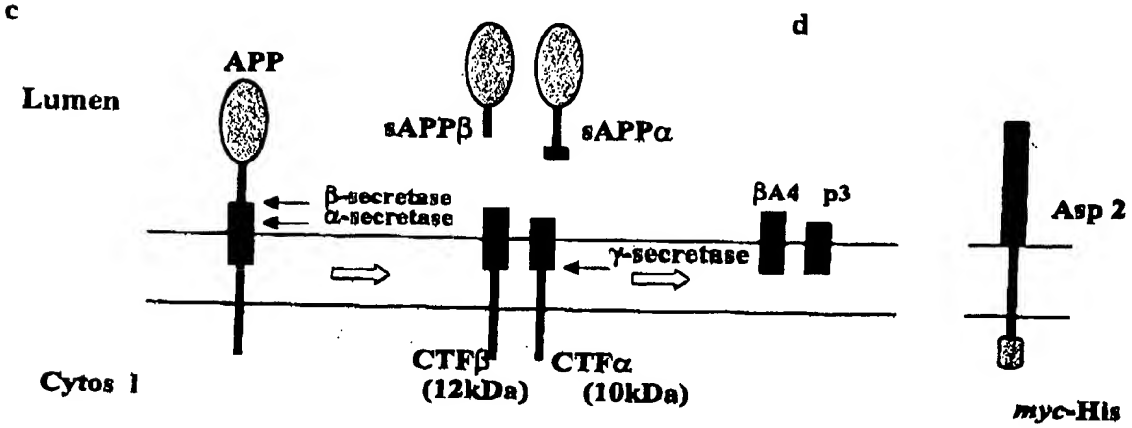

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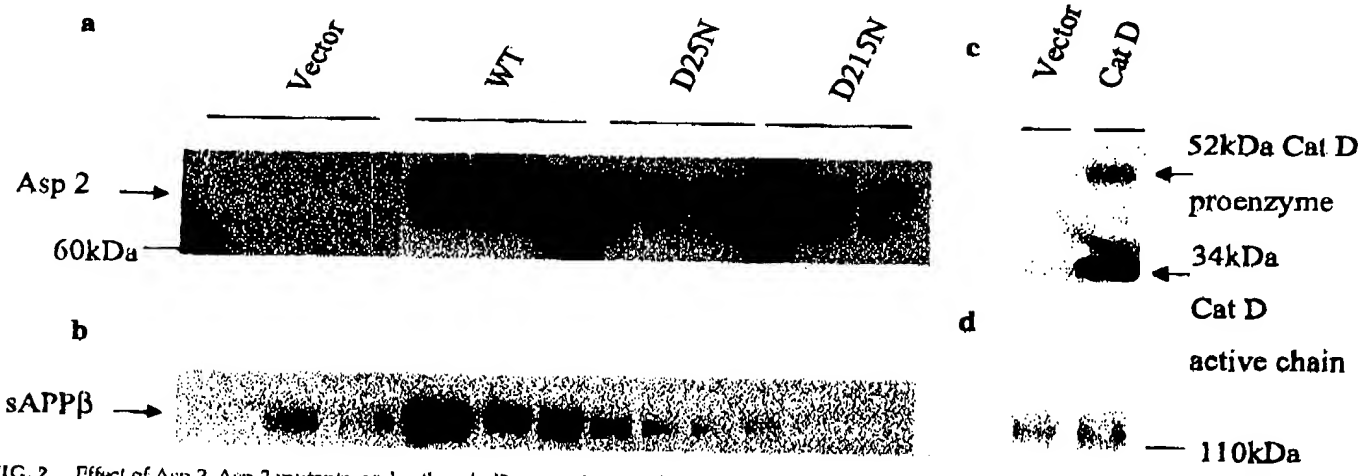

FIG. 2. Effect of Asp 2, Asp 2 mutants, and cathepsin D expression on sAPPβ secretion. Cells were transfected in triplicate and harvested 48 h later for Western blot analysis with an anti-His antibody or an anti-cathepsin D antibody which recognizes the 52-kDa proenzyme and a 34-kDa component of the active proteinase. (a) Expression of the transfected proteins in the SH-SY5Y APP-695 cells. The proteins are expressed at comparable levels. (b) Increase in sAPPβ (~110 kDa) in the medium from the wild-type Asp 2-transfected cells (WT) compared to vector-transfected control cells. There is no increase in sAPPβ secreted from cells transfected with either of the Asp-Asn mutants of Asp 2 (D25N or D215N). (c) Expression of cathepsin D (Cat D) in SH-SY5Y APP-695 cells. (d) There is no increase in sAPPβ secretion from cells transfected with cathepsin D compared to vector.

the C-terminal fragment produced by the action of α-secretase. We have been unable to detect the C-terminal fragment produced by the action of γ-secretase. This may be due to the low levels of this fragment or its rapid degradation in the cell.

To confirm that the increase in production of CTFβ is due to the proteolytic activity of Asp 2, we investigated the effect of expressing the active-site mutants on the levels of CTFβ in COS-7 APP-751 cells. The expression levels of wild-type Asp 2 and the D25N and D215N mutants in the COS-7 APP-751 cells differ in that the expression of the two Asp 2 mutants is slightly lower than that of wild-type Asp 2 (Fig. 4a). However, while there is a significant increase in the production of CTFβ in the presence of wild-type Asp 2, there is no increase in the presence of the mutant enzymes (Fig. 4b). The identity of CTFβ is again confirmed by immunodetection with WO2 (Fig. 4c). Prolonged exposures of the immunoblots failed to reveal the production of any CTFβ in the presence of mutant Asp 2 (data not shown).

We have examined the distribution of Asp 2 in AD hippocampus using a polyclonal antiserum raised to a peptide sequence derived from Asp 2 (see Experimental Methods). We see clear neuronal staining but there is no staining associated with astrocytes, microglia, or oligodendrocytes (Figs. 5a and 5b). While some neurons appear to be more intensely labeled than others, they all show a similar staining pattern with the immunoreactivity localizing to the cytoplasm of the perikaryon and dendrite only. The dendritic staining rarely extends beyond 10–15 μm and no axonal labeling is observed. Within the positive cell bodies themselves, the staining is nonuniform showing slight granularity. Intraneuronal staining is also evident in frontal and temporal cortex and in brain from aged control subjects (data not shown).

Using the same anti-peptide sera we can detect expression of endogenous Asp 2 in SH-SY5Y cells stably expressing the 695 isoform of APP (SH-SY5Y APP-695) and in COS-7 cells expressing the 751 isoform of APP (COS-7 APP-751). The level of Asp 2 is increased upon transient transfection with the protein. Endogenous and transiently expressed Asp 2 is present as a major band at 65 kDa. Upon prolonged exposure of the immunoblot a weaker band at 60 kDa is evident. Deglycosylation experiments reveal that these two bands are differentially glycosylated forms of the same protein (data not shown).

Consistent with several reports (Kuentzel *et al.*, 1993; Walter *et al.*, 1996), APP clearly localizes to the Golgi/endoplasmic reticulum region as revealed by distinctive juxtanuclear staining and a more generalized reticular staining throughout the cell (Figs. 6b and 6g). Asp 2 shows essentially the same subcellular distribution as revealed by simultaneous detection of *myc*-tagged Asp 2 and APP in COS-7 APP-751 cells (compare Figs. 6f and 6g), and merging of the confocal images for APP and Asp 2 indicates colocalization (Fig. 6h). Colocalization is indicated by a yellow–orange signal, the relative levels of the two original signals determining the final color (Fig. 6h). Interestingly, the colocalization is not absolute; APP can be detected more readily toward the cell periphery than Asp 2 (uppermost cell in Figs. 6b, 6g, and

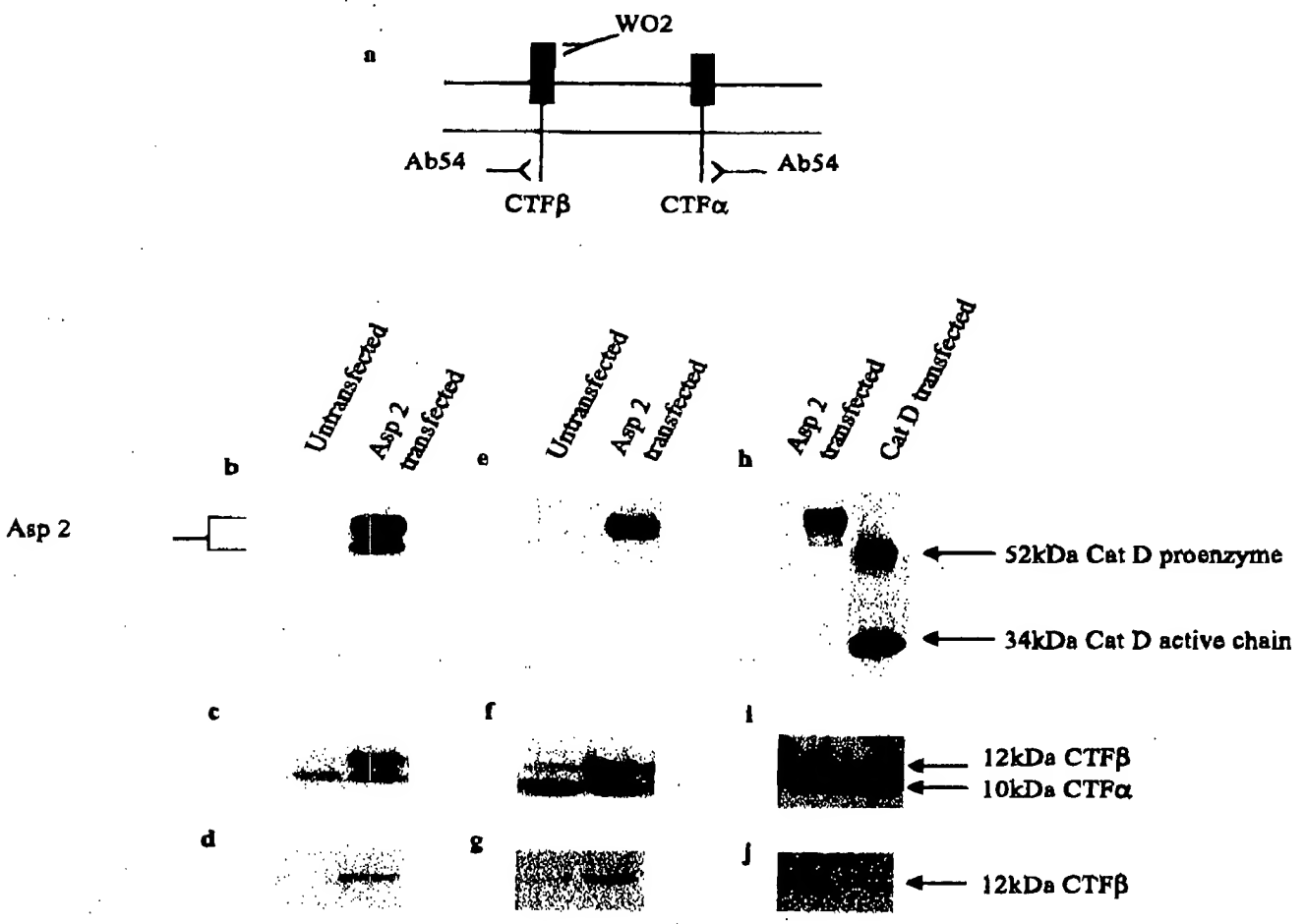

FIG. 3. Effect of Asp 2 and cathepsin D expression on APP CTF production. Cells were transfected in triplicate and lysed 48 h later for Western blot analysis with an anti-His antibody or an anti-cathepsin D antibody. (a) Schematic diagram showing the detection of the APP CTFs by the antibodies used in these studies; Ab54 recognizes CTFα and CTFβ, whereas WO2 only recognizes CTFβ. (b and e) Expression of Asp 2 in COS-7 APP-751 cells with the Swedish mutation or COS-APP-751 cells, respectively, compared to vector. h. Expression of cathepsin D (Cat D) in COS-7 APP-751 cells compared to Asp 2. (c, f, and i) CTFs detected by Ab54, an anti-C-terminal APP antibody which recognizes CTFβ (12 kDa) and CTFα (10 kDa). Transfection with Asp 2 results in an accumulation of the 12-kDa CTF in COS-7 APP-751 Swedish mutant cells (c) and in COS-7 APP-751 cells (f). There is no such accumulation of CTFβ in cells transfected with cathepsin D (i). (d, g, and j) Increase in CTFβ in the Asp 2-transfected cells compared to vector-transfected or cathepsin D-transfected cells as detected with antibody WO2 (recognizes residues 5–8 of human Aβ).

6h). This suggests that these two proteins may segregate as they are processed and transported within the cell. The distribution of both of these proteins is quite distinct from the distribution of markers that define the early endosome (Fig. 6d) and the endoplasmic reticulum (Figs. 6a and 6c).

DISCUSSION

Here we report the characterization of a novel transmembrane aspartic protease which can function in the β-secretase cleavage pathway of APP. The proteinase has many of the expected characteristics of β-secretase, in that it is present in the brain and colocalizes with APP in the Golgi/endoplasmic reticulum of cells. Transfection of Asp 2 into APP-expressing cells results in an increase in the β-secretase activity in cells, such that more sAPPβ is secreted into the medium and there is an accumulation of the β-secretase-derived C-terminal fragment in cells. To show that Asp 2 encodes a functioning protease in cells, we have mutated the two putative active-site aspartic residues. Expression of these mutant Asp 2 proteins did not result in

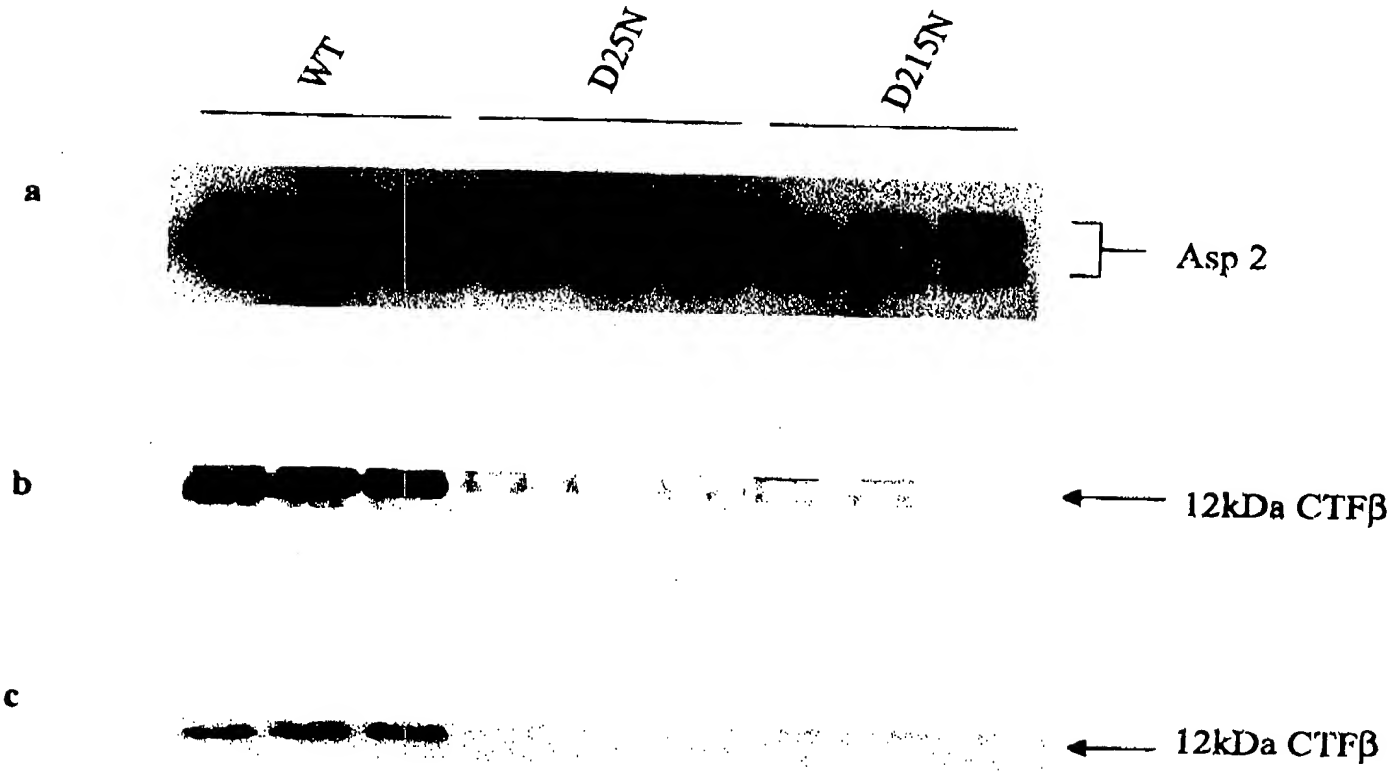

FIG. 4. Effect of Asp 2 and Asp 2 mutants on APP CTF production. Cells were transfected and lysed 48 h later for Western blot analysis. (a) Expression levels of the transfected proteins in COS-7 APP-751 cells detected with an anti-His antibody. (b) Increase in CTFβ in the Asp 2-transfected cells (WT) as detected with Ab54. There is no increase in CTFβ in cells transfected with either of the Asp-Asn mutants (D25N or D215N). (c) Increase in CTFβ in the Asp 2-transfected cells, but not in the mutant Asp 2-transfected cells as detected by antibody WO2.

either an increase in sAPPβ secretion or accumulation of CTFβ. This confirms that expression of Asp 2 produces a functional protease that requires the presence of both catalytic aspartic residues. Our data suggest that γ-secretase is rate limiting in the expression systems we have used as we observe a significant increase in CTFβ, the substrate for γ-secretase. In addition, it has been reported (Uormoneit *et al.*, 1998) that APP bearing the Swedish mutation results in an accumulation of intracellular C-terminal amyloidogenic fragments which may be important in the pathogenesis of AD. Asp 2 may be β-secretase itself or it could act upstream of β-secretase as part of a proteolytic cascade leading to the production of Aβ. However, we currently favor the notion that Asp 2 is β-secretase. Whatever the precise role of Asp 2 in the β-secretase cleavage pathway of APP, our results would suggest that inhibition of its proteolytic activity represents a useful therapeutic target in the treatment of AD.

EXPERIMENTAL METHODS

Plasmids and Transfections

Asp 2 was cloned into pcDNA3.1*myc*-His for transient expression in the mammalian cells. Cathepsin D was expressed using a pBEH-pac18 vector. Asp 2 active-site mutants (D25N and D215N) were constructed with the Stratagene QuickChange mutagenesis kit. Mutants were sequenced to ensure the presence of the desired active-site mutation. For transient transfection, SH-SY5Y APP-695 cells and COS-7 APP-751 or COS-7 APP-751 cells with the Swedish mutation were transfected using Lipofectamine Plus reagent (Life Technologies) as described by the manufacturer. Twenty-four hours posttransfection, the medium was changed to OptiMEM-1 (10 ml). The medium was collected 24 h later, centrifuged briefly (500*g* for 10 min) to remove any cells, and then concentrated using Centriprep 10 concentrators (Amicon). Cells were harvested and lysed by

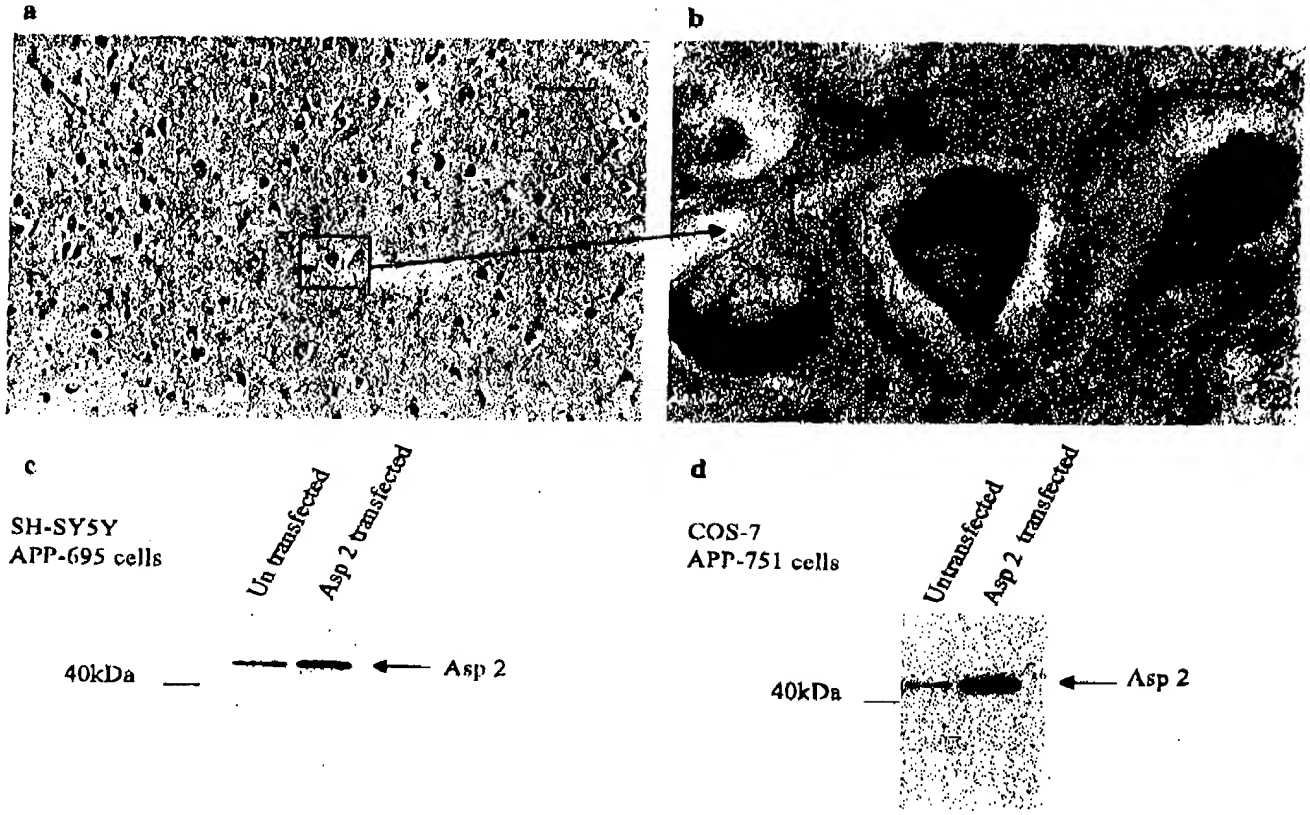

FIG. 5. Demonstration of Asp 2 immunoreactivity in human hippocampus. Sections of tissue from an Alzheimer's disease patient (female, age 62) were labeled with a protein A-purified polyclonal antiserum raised to sequence 121–130 of Asp 2. Scale bars represent 100 μm in (a) and 15 μm in (b). Expression of Asp 2 in (c) SH-SY5Y APP-695 cells and in (d) COS-7 APP-751 cells, respectively. Cells were untransfected or transfected with Asp 2 and harvested 48 h posttransfection for Western blot analysis with the anti-Asp 2 peptide antiserum.

incubation for 30 min at 4°C in 50 mM Tris–HCl, pH 7.4, 1% Triton X-100 containing a cocktail of protease inhibitors (Boehringer-Mannheim). After centrifugation (3000*g* for 5 min), the supernatant was aspirated and stored at −20°C until assayed.

Immunoblotting

Proteins in the cell lysate (20 μg) or media (15 μl of 20-fold concentrated media) were resolved on 10% Tris–glycine or 10–20% Tris–tricine–SDS–polyacrylamide gels (Novex). Polyvinylidene fluoride (PVDF) or nitrocellulose immunoblots were probed with an anti-His_6 antibody (Boehringer-Mannheim), antibody Ab54 raised to the C-terminus of APP, antibody WO2 (raised to amino acids 1–16 of the Aβ domain of APP), antibody 1A9 (raised to the neoepitope region of soluble APP generated after cleavage by β-secretase; LeBrocque *et al.*, 1998), or a monoclonal antibody to human cathepsin D (Calbiochem). Protein A-purified rabbit polyclonal antiserum to a KLH conjugate of Asp 2 sequence RDL-RKGVYEP (amino acid sequence 121–130) was also used to detect Asp 2 in cells. Bound antibody was detected using a peroxidase-conjugated secondary antibody (Sigma) and with an additional peroxidase anti-peroxidase antibody for the membranes probed with antibody 1A9 in conjunction with the enhanced chemiluminescence (ECL) detection method (Amersham).

Subcellular Localization

A cDNA encoding Asp 2 with a *myc* epitope tag was transfected into COS-7 APP-751 cells. Cells were fixed and processed for indirect immunofluorescence as described (Spector, *et al.*, 1998). APP was visualized using the anti-C-terminal antibody Ab54. Control

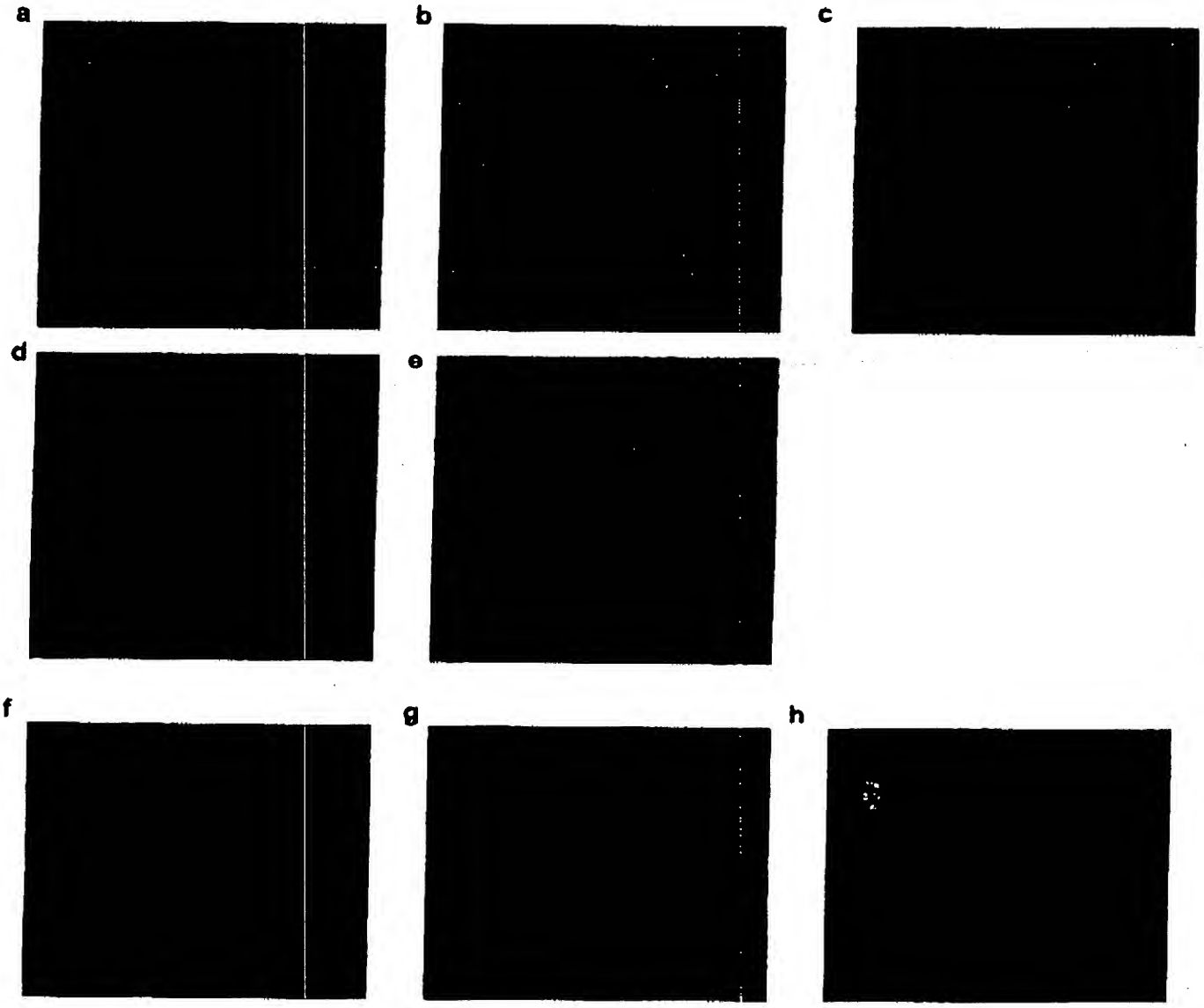

FIG. 6. Subcellular localization of Asp 2 and APP. *myc* epitope-tagged Asp 2 was transfected into COS-7 APP-751 cells. Antigens were detected using antibodies specific for *myc*, APP, and a number of markers for defined subcellular compartments (see Experimental Methods). (a) 58K Golgi protein. (b) APP. (c) Asp 2. (d) Early endosome antigen 1 (EEA1). (e) KDEL. (f) Asp 2. (g) APP. (h) Merged image of f and g showing significant colocalization (yellow) of Asp 2 and APP.

antibodies were monoclonal anti-Golgi 58K protein from Sigma, monoclonal early endosome antigen 1 (EEA1) from Transduction Labs, monoclonal anti-KDEL from Stressgen, and monoclonal anti-*Myc* from Santa Cruz Biotechnology. Detection was with Alexa 488-labeled anti-mouse or Alexa 568-labeled anti-rabbit antibodies from Molecular Probes. Microscopy was carried out using a Leica confocal microscope.

Immunohistochemistry

Ten-micrometer sections of paraformaldehyde-fixed hippocampus and frontal and temporal cortex from two Alzheimer's disease patients (female, ages 62 and 89) and two aged controls (female, ages 80 and 86) were rehydrated and labeled with the polyclonal antiserum to Asp 2 by incubation overnight at 4°C. Subsequent processing used the biotin–avidin sys-

tem (with biotinylated goat anti-rabbit antibody) and the chromogen diaminobenzidine (Vector Laboratories).

ACKNOWLEDGMENTS

We thank A. Hasilik (Institute for Physiologische Chemie, Marburg, Germany) for the cathepsin D construct, K. Walsh and I. Dingwall for technical assistance, D. Dewar (Wellcome Surgical Institute, Glasgow, Scotland) for the AD brain sections, S. Newman for assistance with microscopy, M. Evans for help with confocal microscopy, and Mick Browne. We also thank S. E. Moore for comments on the manuscript.

Note added in proof. While our work was under review, Vassar and colleagues (*Science* 286: 735–741) published a report of β-secretase activity associated with a transmembrane aspartyl proteinase that they designate BACE. Our data indicate that Asp 2 and BACE are the same enzyme.

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Received October 22, 1999
Revised October 26, 1999
Accepted October 25, 1999